

(Supporting Information)

**Nano-flares: probes for transfection, RNA visualization, and detection of transcripts
in living cells**

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Experimental Section

Oligonucleotide synthesis. Oligonucleotides were synthesized on an Expedite 8909 Nucleotide Synthesis System (ABI) using standard solid-phase phosphoramidite methodology. Bases and reagents were purchased from Glen Research. Oligonucleotides were purified by reverse-phase high performance liquid chromatography (HPLC). Sequences used to prepare and test survivin nano-flares are giving in Fig. 1c. Additional oligonucleotide sequences used in this study were: Control particle recognition, 5'-CTA TCG CGT ACA ATC TGC AAA AA-SH-3'; Control particle reporter, 5'-Cy5-TGC AGA TTG TAC G-3'; Survivin molecular beacon, 5'-Cy5-CGA CGG AGA AAG GGC TGC CAC GTC G dabcy1-3'; Control molecular beacon, 5'-Cy5-CGA CGT CGC GTA CAA TCT GCC GTC G-dabcy1-3'.

Preparation of “nano-flare” probes. Citrate-stabilized gold nanoparticles (13 ± 1 nm) were prepared using published procedures.¹ Thiol-modified oligonucleotides were added to 13 ± 1 nm gold colloids at a concentration of 3 nmol of oligonucleotide per 1 mL of 10 nM colloid and shaken overnight. After 12 hours, sodium dodecylsulfate (SDS) solution (10%) was added to the mixture to achieve a 0.1 % SDS concentration. Phosphate buffer (0.1 M; pH = 7.4) was added to the mixture to achieve a 0.01 M phosphate concentration, and six aliquots of sodium chloride solution (2.0 M) were added to the mixture over an eight-hour period to achieve a final sodium chloride concentration of 0.15 M. The mixture was shaken overnight to complete the functionalization process. The solution containing the functionalized particles was centrifuged (13,000 rpm, 20 min) and resuspended in phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Phosphate, 2.7

mM KCl, pH 7.4, Hyclone) three times to produce the purified Au NPs used in all subsequent experiments. The concentration of the particles was determined by measuring their extinction at 524 nm ($\epsilon = 2.7 \times 10^8 \text{ L mol}^{-1} \text{ cm}^{-1}$). Purified oligonucleotide functionalized Au NPs were suspended to a concentration of 10 nM in PBS containing 100 nM of the complementary Cy5 labeled reporter sequence. The mixture was heated to 70 °C, slowly cooled to room temperature, and stored in the dark for at least 12 hours to allow hybridization. Particles were filter sterilized using a 0.2 μm acetate syringe filter (GE Healthcare).

Fluorescence experiments. Nano-flare probes or molecular beacons were diluted to a concentration of 1 nM in PBS containing 0.1 % tween 20 (Sigma) and treated with a complementary target (target concentration, 1 μM). The fluorescence spectra were recorded on a Jobin Yvon Fluorolog FL3-22 exciting at 633 nm and measuring emission from 650 to 750 nm in 1 nm increments.

Nuclease assay. Nano-flare probes were diluted to a concentration of 2.5 nM in PBS (pH 7.0), 0.25 mM MgCl_2 and 50 mg/L Bovine Serum Albumin (Fischer Scientific). Bovine Pancreatic DNase I (United States Biochemical) was added immediately before reading (concentration, 0.38 mg/L). All experiments were performed on a Photal Otsuka Electronics FluoDia T70 with excitation at 620 nm and emission at 665 nm. Molecular beacons were tested in an analogous manner at a concentration of 25 nM. The approximate rates of degradation under these experimental conditions were determined

from the slope of the linear region of the degradation curves (See reference 17 in the Manuscript).

Cell culture and particle incubation. SKBR3 human breast cancer and C166 mouse endothelial cells were obtained from the American Tissue Culture Collection (ATCC) and were grown in McCoy's 5A Medium and Dulbecco's modified Eagles medium (DMEM), respectively, with 10 % heat inactivated fetal bovine serum and maintained at 37 °C in 5 % CO₂. Cells were seeded in 6 or 24 well plates and grown for 1-2 days prior to treatment. On the day of treatment, the cells were approximately 50% confluent. The media was replaced with fresh media containing the functionalized Au NPs. Cells were counted and measured for viability using a Guava EasyCyte Mini (Guava Technologies). Viability after incubation was > 98%.

Imaging. Cells were grown on glass coverslips placed at the bottom of 6 well tissue culture plates. After 1 day, the media was replaced with media containing nano-flares (particle concentration, 125 pM). After 6 hours of treatment, the media was replaced, and the cells were cultured for an additional 12 hours. The coverslips were removed, washed with PBS, and fixed to a chamber filled with PBS mounted on a glass slide. All images were obtained with a Zeiss 510 LSM at 63x magnification using a 633 nm HeNe laser excitation source.

Flow cytometry. Cells were treated with nano-flares as described above (particle concentration, 10 pM). Molecular beacon probes were delivered to cells using

Lipofectamine 2000 (Invitrogen). Cells treated with survivin nano-flares produced 55 times greater fluorescence signal than those treated with survivin molecular beacons and Lipofectamine and thus molecular beacons had to be transfected at 50 times higher concentration (500 pM) for comparison experiments. After treatment, cells were detached from culture flasks using trypsin. Flow cytometry was performed using a DakoCytomation CyAn, exciting at 635 nm.

siRNA delivery and particle incubation. siRNA against human survivin (Santa Cruz) was delivered to cells using Lipofectamine 2000 (Invitrogen) when cells were approximately 50 % confluent (siRNA concentrations, 20, 40, and 80 nM). After 24 hours, the media was exchanged with media containing the nano-flare probes (particle concentration, 50 pM). After 6 hours, the cells were washed and fresh media was added. Cells were cultured for an additional 12 hours and analyzed using flow cytometry.

Quantitative RT PCR. Total RNA was isolated from the cell using an RNeasy Plus Kit (Qiagen) following the manufacturers protocol. During the cell lysis step, 5×10^7 copies of eGFP RNA were added to each sample to account for RNA loss during isolation and purification. To generate RNA standard curves for qRT-PCR, the fragments of RNA to be quantified were generated from the appropriate cellular RNA. Using PCR and primers containing a T7 promoter site, we converted the fragments into transcription compatible sequences (DNA \rightarrow RNA). The transcripts were purified using the MEGAclear kit (Ambion) following the manufacturer's protocol. RNA concentration was determined using the Ribogreen RNA quantification kit (Invitrogen), and a dilution series of stock

RNA was used to generate a standard curve. Primers were: eGFP forward, 5'-TCT TCT TCA AGG ACG ACG GCA ACT-3'; eGFP reverse, 5'- TGT GGC GGA TCT TGA AGT TCA CCT -3'; T7 eGFP forward, 5'-TGC ATA ATA CGA CTC ACT ATA GGG AGA TCT TCT TCA AGG ACG ACG GGC AAC T - 3'; Survivin forward, 5'-ATG GGT GCC CCG ACG TTG-3'; Survivin reverse, 5'- AGA GGC CTC AAT CCA TGG -3'; T7 survivin forward, 5'-TGC ATA ATA CGA CTC ACT ATA GGG AGA TGG GTG CCC CGA CGT TG-3'.

Quantitative-PCR and analysis were performed using LightCycler RNA master SYBR green kits (Roche Applied Sciences) according to the manufacturer's recommendation. Reverse transcription was allowed to proceed at 61 °C for 20 minutes, followed by 45 amplification cycles (95 °C, 5 sec; 54 °C, 15 sec; 72 °C, 20 sec). Target gene RNA was normalized to the standard curves. All reactions were done in triplicate.

Supporting Figures

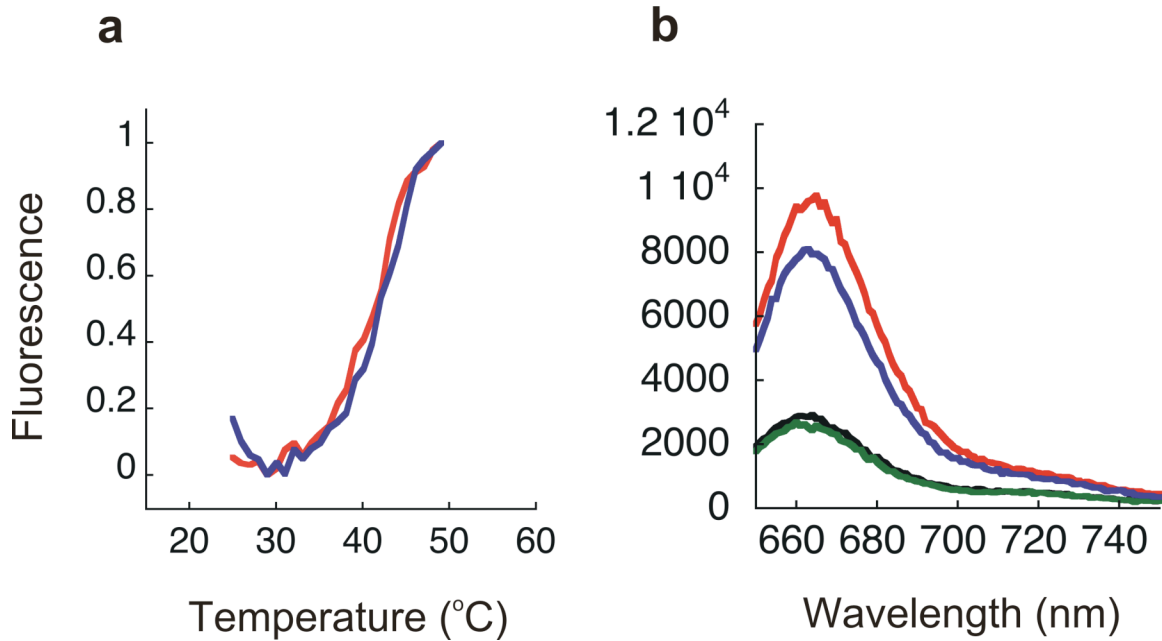


Figure S1. Melting and fluorescence properties of 1 nM survivin and non-complementary nano-flares. (a) Fluorescence melting curves of survivin (red trace) and non-complementary (blue trace) nano-flares. (b) Fluorescence signal of survivin (black trace) and non-complementary (green trace) nano-flares alone. Fluorescence signal of survivin (red trace) and non-complementary (blue trace) nano-flares in the presence of their target sequences (target concentration, 1 μ M). These measurements demonstrate that both the survivin and non-complementary (control) nano-flares have very similar signaling and thermal stability properties.

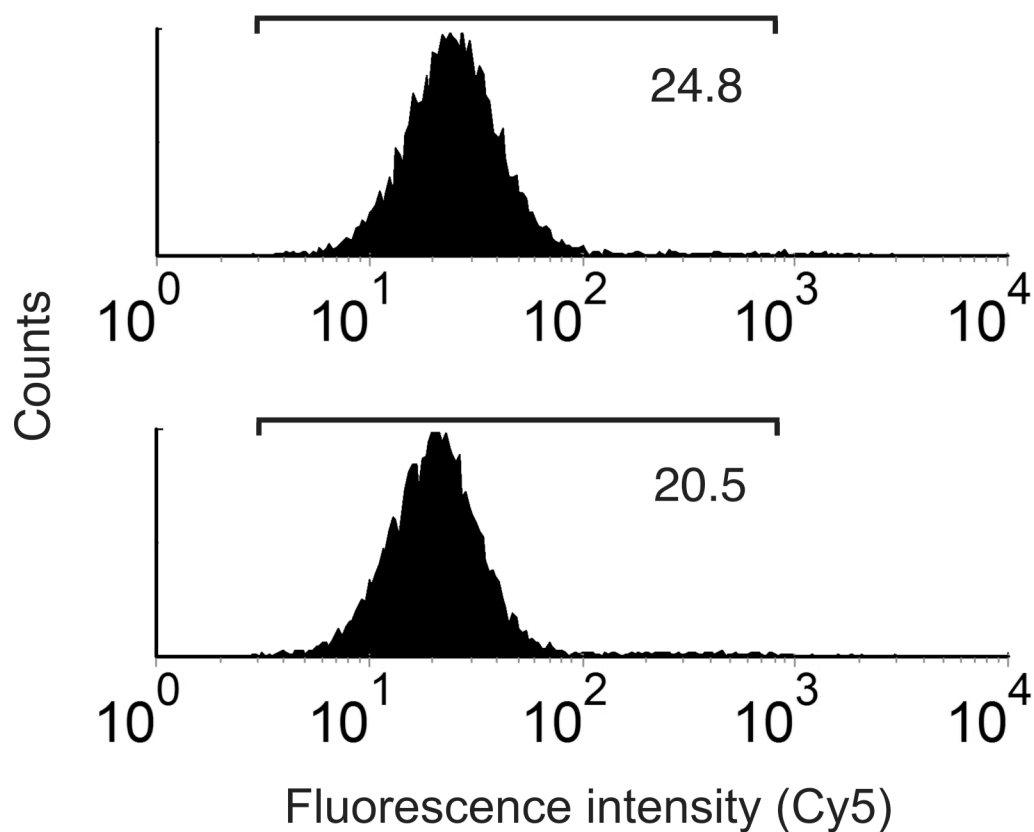


Figure S2. Flow cytometry data collected on SKBR3 cells treated with survivin (top) and non-complementary (bottom) molecular beacons. Mean fluorescence of the population is given in the graph to the left of the histogram. The cells treated with the non-complementary beacons have approximately two-fold higher non-specific fluorescence than those treated with non-complementary nano-flares.

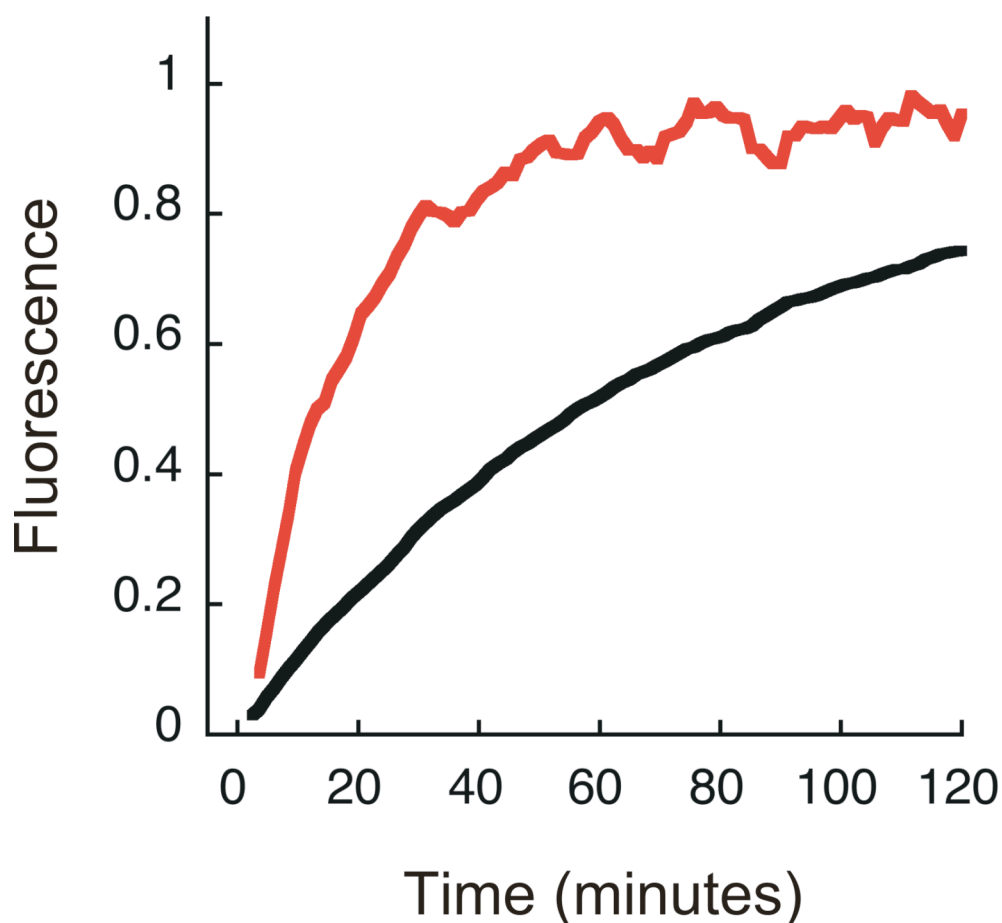
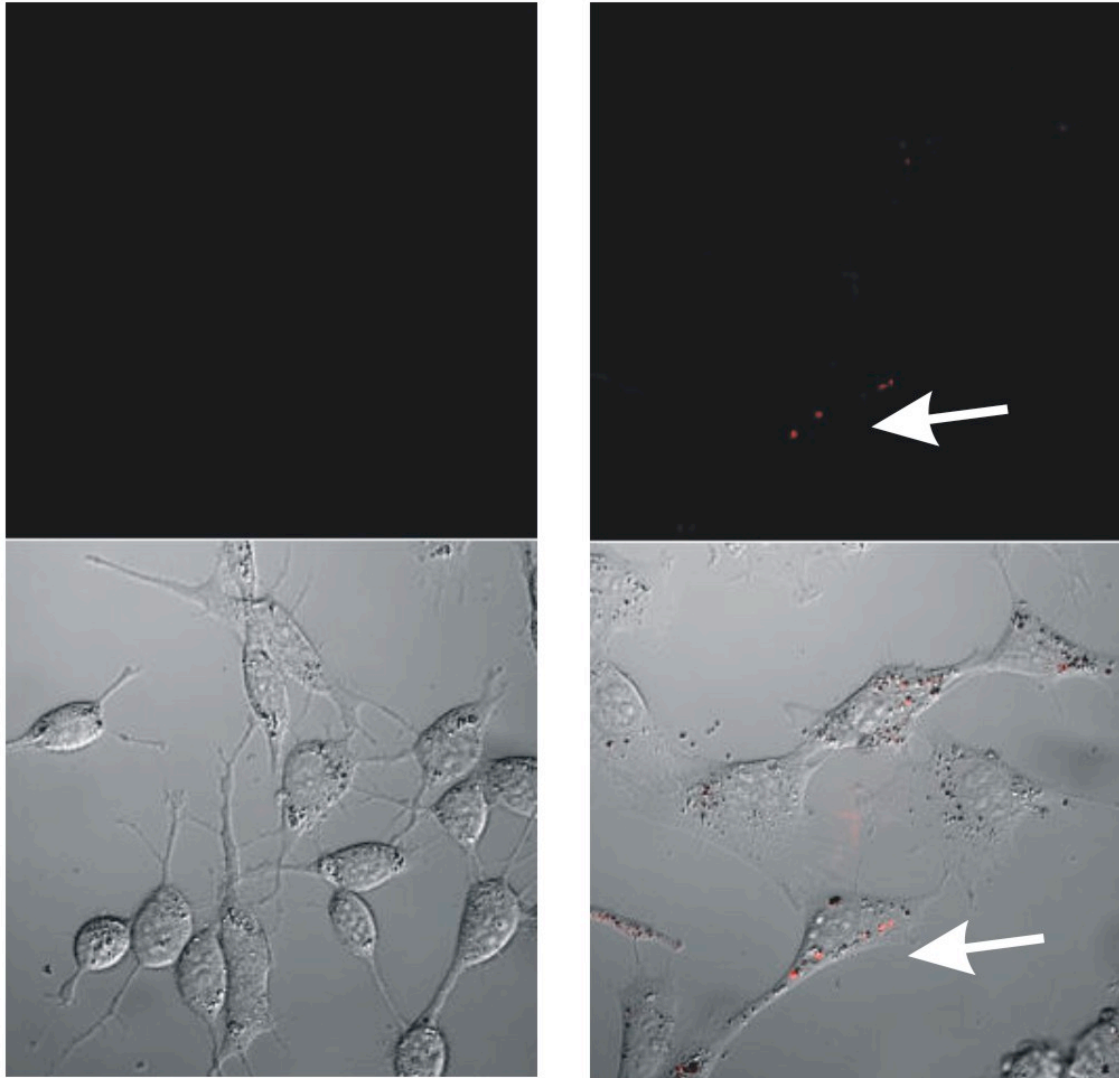


Figure S3. Enzymatic degradation of nano-flares and molecular beacons. Nano-flares (2.5 nM) and molecular beacons (25 nM) were treated with the enzyme DNAase I (0.38 mg/L). The fluorescence signal was recorded as a function of time (nano-flares, black curve; molecular beacons, red curve). In these experiments the concentrations of the probes to was adjusted equalize the number of fluorescent oligonucleotides in solution. The concentration of nano-flares was reduced by a factor of 10 compared to the molecular beacon (Since each nano-flare particle contains 10 fluorescent reporters). Under these conditions, the stoichiometry of enzyme to fluorescent reporter was the same for both probes.



Survivin
Nano-Flare

Non-Complementary
Nano-Flare

Figure S4. Confocal fluorescence images of C166 cells treated with nano-flares at high concentrations (4 nM) to increase the background signal. At these elevated concentrations, control nano-flares show a similar cell or even greater cell-associated fluorescence than the survivin nano-flares.

References

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- ¹ Frens, G. Controlled Nucleation for Regulation of Particle-Size in Monodisperse Gold Suspensions. *Nature-Physical Science* **241**, 20-22 (1973).